Biofilm bacteria: formation and comparative susceptibility to antibiotics

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Abstract

The Calgary Biofilm Device (CBD) was used to form bacterial biofilms of selected veterinary gram-negative and gram-positive pathogenic bacteria from cattle, sheep, pigs, chicken, and turkeys. The minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) of ampicillin, ceftiofur, cloxacillin, oxytetracycline, penicillin G, streptomycin, tetracycline, enrofloxacin, erythromycin, gentamicin, tilmicosin, and trimethoprim-sulfadoxine for gram-positive and -negative bacteria were determined. Bacterial biofilms were readily formed on the CBD under selected conditions. The biofilms consisted of micro-colonies encased in extracellular polysaccharide material. Biofilms composed of *Arcanobacterium (Actinomyces) pyogenes, Staphylococcus aureus, Staphylococcus hyicus, Streptococcus agalactiae, Corynebacterium renale,* or *Corynebacterium pseudotuberculosis* were not killed by the antibiotics tested but as planktonic bacteria they were sensitive at low concentrations. Biofilm and planktonic *Streptococcus dysgalactiae* and *Streptococcus suis* were sensitive to penicillin, ceftiofur, cloxacillin, ampicillin, and oxytetracycline. Planktonic *Escherichia coli* were sensitive to enrofloxacin, gentamicin, oxytetracycline and trimethoprim/sulfadoxine. Enrofloxacin and gentamicin were the most effective antibiotics against *E. coli* growing as a biofilm. *Salmonella* spp. and *Pseudomonas aeruginosa* isolates growing as planktonic populations were sensitive to enrofloxacin, gentamicin, ampicillin, oxytetracycline, and trimethoprim/sulfadoxine, but as a biofilm, these bacteria were only sensitive to enrofloxacin. Planktonic and biofilm *Pasteurella multocida* and *Mannheimia haemolytica* had similar antibiotic sensitivity profiles and were sensitive to most of the antibiotics tested. The CBD provides a valuable new technology that can be used to select antibiotics that are able to kill bacteria growing as biofilms.

Résumé

Le dispositif à biofilm de Calgary (CBD) fut utilisé afin de favoriser la formation de biofilm par différentes bactéries pathogènes à Gram positif ou négatif provenant de bovins, moutons, porcs, poulets et dindes. Les concentrations minimales inhibitrices et les concentrations minimales éliminant le biofilm furent déterminées pour l'ampicilline, le ceftiofur, la cloxacilline, l'oxytétracycline, la pénicilline G, la streptomycine, la tétracycline, l'enrofloxacine, l'érythromycine, la gentamicine, le tilmicosin, et le trimethoprime-sulfadoxine. Dans les conditions appropriées, les biofilms bactériens se formèrent facilement sur le CBD. Les biofilms étaient constitués de micro-colonies bactériennes englobées dans du matériel extracellulaire polysaccharidique. Les biofilms formés par Arcanobacterium (Actinomyces) pyogenes, Staphylococcus aureus, Staphylococcus hyicus, Streptococcus agalactiae, Corynebacterium renale *ou* Corynebacterium pseudotuberculosis *ne furent pas détruits pas les antibiotiques testés alors que les formes libres des bactéries étaient sensibles à de faibles concentrations. Les biofilms et les formes libres de* Streptococcus dysgalactiae *et* Streptococcus suis *étaient sensibles à la pénicilline, au ceftiofur, à la cloxacilline, à l'ampicilline et à l'oxytétracycline. Les formes libres d'*Escherichia coli *étaient sensibles à l'enrofloxacine, la gentamicine, l'oxytétracycline et le trimethoprime-sulfadoxine. L'enrofloxacine et la gentamicine étaient les antibiotiques les plus efficaces envers* E. coli *poussant dans un biofilm. Les formes libres des isolats de* Salmonella *spp. et* Pseudomonas aeruginosa *étaient sensibles à l'enrofloxacine, la gentamicine, l'ampicilline, l'oxytétracycline et le trimethoprime-sulfadoxine alors que le biofilm de ces bactéries n'était sensible qu'à l'enrofloxacine. Les formes libres et les biofilms de* Pasteurella multocida *et* Mannheimia haemolytica *avaient des profils de sensibilité similaires et étaient sensibles à la plupart des antibiotiques éprouvés. Le CBD fournit une nouvelle technologie utile qui peut être utilisée pour sélectionner les antibiotiques qui sont en mesure de détruire les bactéries capable de croître dans un biofilm.*

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Introduction

Historically, we have studied these microorganisms by culturing them in highly enriched liquid or solid media that artificially selects for less hardy bacteria (1–3). However, bacteria exist within natural systems in an entirely different form from these artificially grown laboratory strains (1–3). In order for bacteria to survive within hostile environments such as that encountered in host tissue (antibodies, phagocytes, etc.) or on an inert surface exposed to inhospitable conditions (UV light, desiccation, heat, cold, shear

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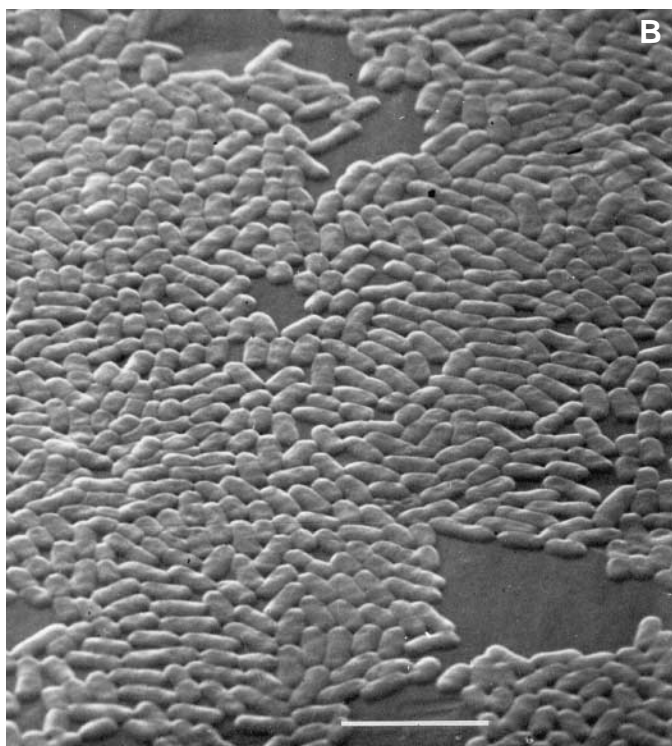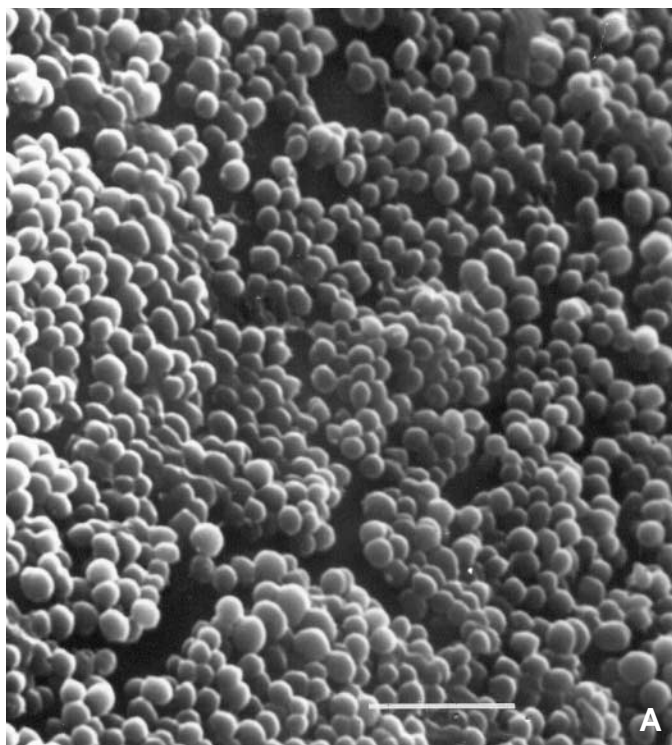
Figure 1. Representative examples of biofilm formation of veterinary pathogens. *Staphylococcus aureus* (mastitis isolate), *Pasteurella multocida* (poultry isolate), and *Corynebacterium renale* (bovine isolate) are demonstrated colonizing the peg of the CBD in Figure 1a, 1b, and 1c, respectively. Note how the bacteria tend to grow in clumps (microcolonies) and the exopolysaccharide that is covering the bacteria; bar = 5 μm.

forces), they have adapted by existing as adherent populations (sessile bacteria). Sessile bacteria appear to be protected in these antagonistic environments by growing as colonies encased in an extracellular matrix of carbohydrate or exopolysaccharide (1–4). A large collection of these groups of bacterial cells adhering to a surface is called a bacterial biofilm (1–3). When bacteria are examined in natural environments and within infected tissue, biofilms are the most predominant form. Sessile bacteria growing on surfaces have nutrient limitations and so may grow more slowly and have restricted mobility (4); planktonic forms in culture media have unnatural access to nutrients, multiply rapidly and often are highly motile. Planktonic bacteria are more susceptible to the effects of antibiotics and to environmental and host factors (1–4). Conversely, sessile bacteria are able to resist or evade such destructive factors by forming aggregates, altering their physiology, and taking advantage of deficiencies in the host clearance mechanisms (1–4).

Many common bacterial pathogens exist in animals as biofilms. Typical animal diseases where bacterial biofilms are believed to be involved based on histopathologic and ultrastructural appearance of the bacteria within tissue include: mastitis (*Streptococcus agalactiae, Staphylococcus aureus*), pneumonia (*Mannheimia haemolytica, Pasteurella multocida*), liver abscess (*Fusobacterium necrophorum*), lymphadenitis (*Corynebacterium pseudotuberculosis, Streptococcus* spp.), enteritis (*Escherichia coli, Salmonella* spp.) and wound infections (*Staphylococcus aureus, Pseudomonas aeruginosa*) (1). Infections that involve a biofilm mode of growth are generally chronic and are often difficult to treat (1–4).

Traditionally, microbiologists have evaluated the efficacy of an antibiotic by measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (5,6). In virtually all diagnostic laboratories, these measurements are made on freely floating, planktonic, laboratory phenotypes. These assays measure only the concentration of chemotherapeutic agent required

Table I. Source, growth conditions, and concentration of sessile (biofilm) and planktonic bacteria

Organism	Source	Time (h)	Growth conditions	Biofilm concentration (cfu/peg)[a]	Planktonic concentration (cfu/mL)[b]
Arcanobacterium pyogenes	Bovine pneumonia	24	TSB + 2% FCS; 10% CO_2	9.0×10^4	5.5×10^9
Escherichia coli 987P	Bovine enteritis	7.5	TSB	1.9×10^6	1.1×10^{10}
Escherichia coli	Bovine enteritis	6	TSB	5.5×10^4	3.5×10^9
Escherichia coli K99	Bovine diarrhea	5	TSB	2.5×10^6	5.0×10^9
Escherichia coli	Turkey enteritis	5	TSB	7.0×10^4	4.0×10^8
Escherichia coli F41	Bovine diarrhea	5	TSB	1.7×10^6	5.0×10^9
Haemophilus somnus	Bovine pneumonia	7	HS medium; 10% CO_2	1.0×10^4	1.0×10^8
Mannheimia haemolytica	Bovine pneumonia	24	TSB + 2% FCS; 10% CO_2	1.0×10^5	1.5×10^9
Pasteurella multocida	Bovine pneumonia	7	TSB + 2% FCS; 10% CO_2	8.5×10^4	2.5×10^{10}
Pasteurella multocida	Chicken cholera	5	TSB + 2% FCS; 10% CO_2	1.5×10^5	5.5×10^9
Pseudomonas aeruginosa	Wound isolate	4	TSB	1.1×10^6	3.0×10^8
Salmonella sp.	Porcine enteritis	5	TSB	1.5×10^5	1.0×10^9
Salmonella Typhimurium	Bovine enteritis	4	TSB	3.0×10^5	1.8×10^9
Salmonella Bredeney	Turkey enteritis	6	TSB	2.0×10^6	2.6×10^{10}
Staphylococcus aureus	Bovine mastitis	7	TSB; 10% CO_2	1.5×10^5	1.5×10^7
Staphylococcus hyicus	Porcine dermatitis	7.5	TSB; 10% CO_2	6.3×10^5	2.2×10^8
Streptococcus bovis	Bovine rumen	7	TSB; 10% CO_2	1.6×10^5	1.7×10^9
Streptococcus suis	Porcine pericarditis	7	TSB; 10% CO_2	6.0×10^4	1.3×10^9
Streptococcus agalactiae	Bovine mastitis	7	TSB; 10% CO_2	1.6×10^5	1.3×10^8
Streptococcus dysgalactiae	Bovine mastitis	7	TSB; 10% CO_2	1.0×10^5	4.5×10^7
Corynebacterium renale	Pyelonephritis	24	TSB + 2% FCS; 10% CO_2	1.5×10^5	2.5×10^7
Corynebacterium pseudotuberculosis	Lymphadenitis	24	TSB + 2% FCS; 10% CO_2	3.0×10^4	2.0×10^6

TSB — tryptic soy broth; FCS — fetal calf serum; HS — *Haemophilus somnus* medium; cfu — colony-forming units

[a] This column represents the mean number of sessile bacteria present on each peg of the Calgary Biofilm Device (CBD)

[b] This column represents the mean number of planktonic bacteria growing in the trough of the CBD at the same time the peg was sampled

to inhibit growth or kill planktonic bacteria (5,6). For some antibiotics, the concentration required to kill sessile bacteria may be greater than a thousand times that required to kill planktonic bacteria of exactly the same strain (4,7). Therefore, the use of typical laboratory planktonic bacteria for selection of chemotherapeutics may be inappropriate under some circumstances.

We have recently developed a technology to screen the effectiveness of antibiotics or biocides at eliminating sessile bacteria in vitro (8). The use of the Calgary Biofilm Device (CBD) permits rapid selection of potentially effective antibiotics for killing sessile bacteria in vivo and biocides for disinfecting contaminated inert surfaces (8). This device determines the minimum biofilm eradication concentration (MBEC), which is the concentration of an antimicrobial agent required to kill a bacterial biofilm. Recent studies conducted in our laboratory have demonstrated that selecting antibiotics that are effective for eliminating bacterial biofilms may improve the success rate in treating clinical and experimentally induced disease (9). The objective of this project was to determine culture conditions where veterinary pathogens would form biofilms on the CBD. A second objective was to evaluate the ability of antibiotics commonly used in veterinary medicine to eliminate a diverse selection of bacterial biofilms. Ultimately, the study was conducted to provide the basis of future extensive screening of the susceptibility of antibiotics to veterinary bacterial pathogens.

Materials and methods

Organisms

Bacterial isolates were obtained from clinical cases of infections of cattle, sheep, pigs, chickens and turkeys. Isolates were obtained from the Animal Health Unit of the University of Calgary in Calgary, Alberta and from Alberta Agriculture, Food and Rural Development in Edmonton, Alberta. These isolates included: *Corynebacterium pseudotuberculosis, Corynebacterium renale, Mannheimia haemolytica, Pasteurella multocida, Arcanobacterium (Actinomyces), Haemophilus somnus, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus hyicus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus suis, Escherichia coli* and *Salmonella* spp.

Selection of antibiotics

The antibiotics evaluated were those commonly approved for the treatment of bacterial infections in animals. The antibacterial agents evaluated included: ampicillin, cloxacillin, erythromycin, gentamicin, oxytetracycline, penicillin G, streptomycin, tetracycline (Sigma Chemical Company, St. Louis, Missouri, USA), ceftiofur (The Upjohn Corporation, Kalamazoo, Michigan, USA), enrofloxacin (Bayer Animal Health, Kansas City, Kansas, USA), tilmicosin (Eli

Table II. Sensitivity of gram-positive planktonic and sessile (biofilm) bacteria to veterinary antibiotics

Bacterium		Penicillin G	Cloxacillin	Streptomycin	Ceftiofur	Tetracycline	Ampicillin	Oxytetracycline
Arcanobacterium	MIC	< 2	< 2	4	< 2	< 2	< 2	< 2
pyogenes	MBEC	> 1024	> 1024	256	> 1024	> 1024	500	> 1024
Staphylococcus	MIC	2	< 2	128	< 2	< 2	32	< 2
aureus	MBEC	> 1024	512	> 1024	256	512	128	128
Staphylococcus	MIC	16	< 2	512	< 2	32	4	64
hyicus	MBEC	> 1024	4	> 1024	128	256	4	250
Streptococcus	MIC	< 2	< 2	64	< 2	< 2	< 2	< 2
agalactiae	MBEC	> 1024	> 1024	256	> 1024	> 1024	> 1024	1024
Streptococcus	MIC	< 2	< 2	32	< 2	< 2	< 2	< 2
dysgalactiae	MBEC	< 2	< 2	64	< 2	4	< 2	8
Streptococcus suis	MIC	< 2	< 2	128	< 2	32	< 2	8
	MBEC	8	< 2	128	< 2	32	< 2	8
Corynebacterium	MIC	< 2	< 2	16	< 2	< 2	< 2	< 2
renale	MBEC	> 1024	> 1024	128	> 1024	1024	> 1024	> 1024
Corynebacterium	MIC	< 2	< 2	256	< 2	< 2	< 2	< 2
pseudotuberculosis	MBEC	> 1024	> 1024	256	1024	256	> 1024	> 1024

MIC — minimum inhibitory concentration; MBEC — minimum biofilm eradication concentration

Lilly, Greenfield, Indiana, USA), and trimethoprim-sulfadoxine (Hoechst Animal Health, Regina, Saskatchewan).

Biofilm formation on the Calgary Biofilm Device

Biofilm formation and measurement of antimicrobial sensitivity of bacterial biofilms were performed on the CBD (MBEC Biofilm Technologies, Calgary, Alberta) according to previously described methods (8). The device features a microtiter plate lid with 96 pegs or projections distributed on the lid. Each peg provided the surface for bacteria to adhere, colonize and form a uniform biofilm (8). The pegs fit precisely into the wells of a standard 96-well microtiter plate. The lid was used in conjunction with special troughs for growing of bacteria, washing, and incubating. One of tryptic soy broth (BDH), tryptic soy broth with bovine serum (Sigma Chemical Company) or HS broth (Difco Laboratories, Detroit, Michigan, USA) for *H. somnus* was placed in the trough. The trough was inoculated with approximately 10^8 test bacteria (based upon McFarlane standards) obtained from colonies selected from tryptic soy agar (TSA, BDH) or brain heart infusion (BHI, BDH) agar plates. The pegged lid was placed over the troughs and the unit incubated on a rocker [Red Rocker; Hoefer Instruments, San Francisco, California, USA; 10 rpm ($2.5 \times g$)] at 37°C and 95% relative humidity. The pegs were colonized for 4 to 24 h (depending on the specific bacterial growth rate). Selection of culture conditions for colonization of the pegs was determined in preliminary studies and the assessment of biofilm was determined by breaking several pegs from various points on the lid. The removed pegs were placed in microfuge tubes containing 200 μL of TSB, sonicated (Aquasonic, model 250; VWR Scientific, Buffalo Grove, Illinois, USA) for 5 min and plate counts of viable bacterial cells were performed on TSA or BHI agar containing 10% sheep blood. Additional pegs were fixed with 2.5% gluteraldehyde in phosphate-buffered saline (PBS), air-dried overnight, and prepared for scanning electron microscopy (SEM) (Hitachi model 450; Hitachi, Tokyo, Japan), as described previously (10).

Minimum biofilm eradication concentration assay

Assays were performed when pegs contained approximately 10^4 to 10^6 bacteria growing as a biofilm following conditions developed from the procedure described above. By using SEM, we have established that biofilms are produced at this level of peg colonization and we are not studying adherent bacterial cells. These biofilms can then be used for assessment of antimicrobial activities. Non-adherent bacteria on the pegs were washed from the pegs in a 96-well microtiter plate containing sterile PBSS. Each test antibiotic was placed in one lane of the microtiter plate at 2-fold dilutions of antibiotic (from 1024 μg/mL to 2 μg/mL). Seven antibiotics were evaluated on each plate and one lane served as a negative control (no antibiotic). All samples were run in duplicate. Pegs with the bacterial biofilm were secured over the test microtiter plate and the plate was incubated for 24 h at 37°C with antibiotic. The pegged lid was then removed, rinsed in PBS, then placed over a second 96-well microtiter plate containing fresh, sterile broth medium. The remaining biofilm was removed from the pegs by ultrasonic disruption for 5 min. This plate was incubated for 24 h at 37°C and the presence of viable bacteria determined by plate counts or turbidity determined at 650 nm in a 96-well plate reader (Molecular Devices; Fisher Scientific, Nepean, Ontario). Growth of bacteria in a particular well indicates regrowth of planktonic bacteria from surviving biofilm. Therefore, the MBEC value represents the lowest dilution at which bacteria fail to regrow.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC), which represents the concentration of antibiotic required to inhibit growth of a planktonic bacterial population, was determined using the CBD. The MIC was determined from the bacteria that were shed from the pegs of the CBD when it was placed in the differing concentrations of antibiotics (8). The MIC values obtained using the CBD are similar

Table III. Sensitivity of gram-negative planktonic and sessile (biofilm) bacteria to veterinary antibiotics

Bacteria		Enrofloxacin	Gentamicin	Erythromycin	Tilmicosin	Ampicillin	Oxytetracycline	Trimeth/Sulfa
Escherichia coli	MIC	< 2	32	256	256	> 1024	4	< 2
(K99 calf enteritis)	MBEC	64	32	> 1024	> 1024	> 1024	> 1024	> 1024
Escherichia coli	MIC	< 2	256	64	128	< 2	8	< 2
(turkey enteritis)	MBEC	64	> 1024	256	512	32	> 1024	> 1024
Escherichia coli	MIC	< 2	8	> 1024	128	> 1024	> 1024	4
(bovine mastitis)	MBEC	128	> 1024	> 1024	> 1024	> 1024	1024	> 1024
Escherichia coli	MIC	< 2	32	1024	128	2	256	4
(F41 calf enteritis)	MBEC	< 2	512	1024	1024	> 1024	512	> 1024
Escherichia coli	MIC	< 2	4	16	64	< 2	128	< 2
(calf enteritis)	MBEC	8	4	256	> 1024	4	250	> 1024
Salmonella sp.	MIC	< 2	16	64	128	< 2	8	512
(porcine enteritis)	MBEC	128	> 1024	> 1024	> 1024	> 1024	> 1024	1024
Salmonella	MIC	< 2	16	32	64	> 1024	128	< 2
Typhimurium	MBEC	< 2	64	> 1024	> 1024	> 1024	256	> 1024
Salmonella Bredeney	MIC	< 2	500	125	125	< 2	64	< 2
(turkey enteritis)	MBEC	4	> 1024	512	> 1024	< 2	256	> 1024
Pasteurella multocida	MIC	< 2	64	4	16	< 2	< 2	< 2
(calf pneumonia)	MBEC	< 2	64	4	16	< 2	< 2	< 2
Pasteurella multocida	MIC	< 2	32	< 2	16	< 2	< 2	< 2
(chicken septicemia)	MBEC	< 2	32	4	16	< 2	< 2	512
Mannheimia haemolytica	MIC	< 2	64	2	< 2	1024	64	< 2
(pneumonia)	MBEC	< 2	64	8	< 2	> 1024	128	32
Pseudomonas aeruginosa	MIC	< 2	256	64	128	> 1024	16	256
(wound isolate)	MBEC	8	1024	512	> 1024	> 1024	> 1024	> 1024
Haemophilus somnus	MIC	< 2	16	< 2	< 2	8	< 2	< 2
(bovine respiratory)	MBEC	< 2	16	< 2	< 2	8	< 2	< 2

MIC — minimum inhibitory concentration; MBEC — minimun biofilm eradication concentration; Trimeth/sulfa — trimethoprim-sulfadoxine (1:5)

to those obtained using the National Committee for Clinical Laboratory Standards (NCCLS) procedure (8).

Results

Bacterial biofilms were readily formed by most pathogens on the CBD (Table I). The biofilms consisted of microcolonies encased in extracellular polysaccharide material (1,4). Typical biofilms are illustrated in Figures 1a, 1b, and 1c. Although bacteria grew readily as planktonic organisms in liquid culture media, some bacteria would not form biofilms under standard cultural conditions. Special conditions were required for these organisms to grow as biofilms (Table I). In order to form biofilms, *C. renale, C. pseudotuberculosis, M. haemolytica, P. multocida,* and *A. pyogenes* required the addition of fetal bovine serum and incubation under 10% CO_2. The duration of heavy biofilm formation on the pegs [> 10^4 colony-forming units (cfu) per peg] varied from 4 h for *P. aeruginosa* to 24 h for *A. pyogenes, M. haemolytica, C. renale,* and *C. pseudotuberculosis*. Bacteria that required these more specialized culture conditions to form biofilms also required longer culture time.

The concentrations of antibiotic required to inhibit planktonic bacteria (MIC) and those required to kill biofilm bacteria (MBEC) are summarized in Tables II and III. Most antibiotics were effective in inhibiting planktonic bacterial growth at low concentrations and the

bacteria would be considered sensitive based upon NCCLS breakpoints (11). Only a limited number of antibiotics were effective in killing biofilm bacteria at relatively low concentrations. In some cases, biofilm bacteria, such as *A. pyogenes* and *S. aureus*, appeared to lack sensitivity to all of the antibiotics evaluated.

Gram-positive organisms growing as biofilms proved to be particularly resistant to most antimicrobial agents. Most planktonically growing organisms were sensitive to virtually all of the antibiotics tested (Table II). Biofilms composed of *A. pyogenes, S. aureus, S. hyicus, S. agalactiae, C. renale,* and *C. pseudotuberculosis* were highly resistant to antimicrobial agents evaluated but sensitive to the tested agents as planktonic bacteria. Both biofilm and planktonic forms of *S. dysgalactiae* and *S. suis* were sensitive to the β-lactam drugs (penicillin, ceftiofur, cloxacillin, ampicillin) and oxytetracycline.

There was considerable variation in the results obtained for *E. coli* isolates grown as sessile (biofilm) and planktonic populations (Table III). Planktonic *E. coli* were sensitive to enrofloxacin, gentamicin, oxytetracycline, and trimethoprim/sulfadoxine. Enrofloxacin and gentamicin were the most effective antibiotics for *E. coli* growing as biofilms. *Salmonella* growing as planktonic populations were sensitive to enrofloxacin, gentamicin, ampicillin, oxytetracycline, and trimethoprim/sulfadoxine. When *Salmonella* spp. were grown as biofilms, they were only sensitive to enrofloxacin and ampicillin (*Salmonella* Bredeney only). Planktonic and biofilm populations of

P. multocida and *M. haemolytica* had similar antibiotic sensitivity profiles with the exception of trimethoprim/sulfadoxine. Planktonic *P. aeruginosa* were sensitive to enrofloxacin, erythromycin, and oxytetracycline. The sessile forms of this organism were sensitive only to enrofloxacin. Both planktonic and sessile *H. somnus* were sensitive to enrofloxacin, gentamicin, erythromycin, tilmicosin, ampicillin, oxytetracycline, and trimethoprim/sulfadoxine.

Discussion

The MIC has been used as a gold standard for determination of antimicrobial sensitivities for animal and human pathogenic bacteria (4,5). It is recognized that an antibiotic that is ineffective in preventing growth of a particular organism using the MIC assay will also be clinically ineffective (12). However, an organism that is sensitive in vitro may not be effective in vivo (12–16). For many veterinary bacterial diseases the MIC value for a particular antibiotic is not predictive of clinical efficacy. Nevertheless, up to this time, the MIC assay remains the best way to select potentially effective antimicrobial agents. The CBD and the MBEC assay were developed for rapid and reproducible antimicrobial susceptibility testing for bacterial biofilms in the anticipation that the MBEC would be more reliable for selection of clinically effective antibiotics.

In human medicine it has been estimated that 65% of nosocomial infections are biofilm associated, costing the health care system billions of dollars (1,3,15). These biofilm infections are 10 to 1000 times more resistant to the effects of antimicrobial agents (1,3,7). Indeed, many veterinary bacterial pathogens exist predominantly as adherent (also called biofilm or sessile) organisms within tissue and on inert surfaces and it is well recognized that such infections are extremely difficult to successfully treat (14,15,17,18). The mechanism for enhanced antimicrobial resistance is believed to involve alterations in gene expression leading to a phenotype difference between the planktonic and sessile forms. The sessile forms are more resistant as they produce exopolysaccharde, have different growth characteristics and take up nutrients and drugs differently from their planktonic counterpart (3,16). The CBD was developed to address the issues of enhanced antimicrobial resistance within biofilms. Determination of MBEC might, therefore, permit selection of a particular antibiotic that would more closely reflect the prognosis of antimicrobial therapy for a particular bacterial infection.

Staphylococcus biofilms have been extensively studied in human medicine and this pathogen is considered significant in both device associated infections and tissue infections such as pneumonia and osteomyelitis (1,19). The prevalence of bovine staphylococcal mastitis ranges from 7% to 40% of all dairy cattle and this infection is associated with bacterial biofilms (14,17–19). It is also recognized that antibiotic therapy may temporarily eliminate clinical signs of mastitis but the prognosis of a complete cure is poor (14). Although, in this study, the MIC assay clearly indicated that many antibiotics should be effective in the treatment of bovine mastitis, the MBEC values data demonstrated that the *S. aureus* isolate is resistant to antibiotics tested, correlating with clinical observations. The *S. hyicus* biofilm as measured by MBEC was sensitive to many of the antibiotics tested; indeed, *S. hyicus* usually responds well to antibiotic therapy (20).

There is considerable variability in therapeutic responses to streptococcal infections (20). *S. suis* and *S. dysgalactiae* infections frequently respond readily to most chemotherapeutic agents (20). Although these organisms formed biofilms, the MIC and MBEC values were similar, suggesting that most of the antibiotics evaluated would be effective as chemotherapeutic agents. The *S. agalactiae* isolate studied, recovered from an animal with chronic mastitis, was sensitive to most antibiotics according to the MIC, but as a biofilm, it was resistant. *S. agalactiae* mastitis is highly infectious and usually responds to treatment. As this isolate was recovered from an animal with chronic unresponsive mastitis, it may be genotypically and phenotypically altered to be resistant as a biofilm (3,16).

Although *C. renale, C. pseudotuberculosis* and *A. pyogenes* grew readily as planktonic bacteria in enriched broth, they required specific culture conditions, such as addition of serum to the media and culturing under increased carbon dioxide concentration, to induce formation of biofilms. This suggests that for some microorganisms simulation of the growth conditions that exist in the host may be required. These organisms were sensitive to all antimicrobial agents tested (except streptomycin) according to the MICs, but they were highly resistant according to the MBEC values. The MBEC values appear to be more predictive, as infections caused by *C. renale, C. pseudotuberculosis,* and *A. pyogenes* require prolonged antimicrobial therapy and are frequently unresponsive to treatment (20). The ability of biofilm bacteria to avoid phagocytosis by macrophages and neutophils may also account for the abscessation observed within these infections (1). The accumulation of pus and the associated encapsulation of the infection site also inhibits the antimicrobial penetration and pathogen destruction.

Most gram-negative livestock pathogens readily form bacterial biofilms and these biofilms have been previously described in livestock infections such as neonatal colibacillosis (21) and pneumonic pasteurellosis (18). The veterinary *E. coli* isolates tested readily formed biofilms on the CBD and with the exception of enrofloxacin, gentamicin, and ampicillin, these biofilms were resistant to the antibiotics tested. This suggests that once *E. coli* biofilms have been established, they may be difficult to treat with some antibiotics. This has been observed in some clinical cases in cattle, swine and poultry (20,22). There was considerable variability among the MICs of the *Salmonella* spp. isolates in this study: similar variability between the MIC and the MBEC values was observed. This observation may reflect the complexity in prediction of chemotherapeutic agents for treatment of different *Salmonella* isolates. Bovine, porcine, and avian *Pasteurella* spp., as well as the *H. somnus* and *M. haemolytica* isolate tested, formed biofilms, but in most cases there was no difference between the MIC and the MBEC values. Indeed, animals with pasteurellosis or hemophilosis respond well to most antimicrobial agents provided that a secondary pathogen (*A. pyogenes, S. aureus*) is not involved (20).

Pseudomonas aeruginosa has been recognized in human medicine to form antibiotic resistant biofilms on implanted devices and within tissues (3). *Pseudomonas aeruginosa* infections in animals are similarly difficult to treat (23). The planktonic *Pseudomonas* isolate was resistant to most antibiotic agents, but biofilm cells were more resistant and only enrofloxacin demonstrated reasonable

clinical activity. Fluorinated quinolones have been shown to be effective in treatment of most *Pseudomonas* infections (1,3).

Planktonic bacterial sensitivity, pharmacokinetics, drug penetration, local activity, and drug inactivation all influence the clinical efficacy of an antibacterial agent, but to date, the efficacy of veterinary antibiotics in elimination of bacterial biofilms has not been evaluated. The CBD and the MBEC assay provide a new technology that can be used to select antibiotics that are effective in killing biofilm bacteria. This new technology can also be used in the pharmaceutical industry for developing new antimicrobial agents with efficacy against bacteria growing as biofilms (8). Recently, we have used the MBEC assay to predict clinical failure and clinical success of certain antibiotics used to treat peritonitis due to device-associated infections in humans (24). It may be possible to apply this technology in veterinary bacterial infections that are difficult to treat. This study was conducted to demonstrate the diversity of organisms that could form biofilms that were resistant to common veterinary antibiotics. Further studies are required to document variations within a specific species or from a defined bacterial disease.

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